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A Hypothesis for the Role of Dithiol-Disulfide Interchange in Solute Transport and Energy-Transducing Processes

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We have recently shown that the physical mechanism for $\Delta\tilde{\mu}_{H^+}$-driven changes in the K_m for three different transport systems is an oxidation-reduction reaction involving a dithiol-disulfide interconversion [Robillard, G. T. and Konings, W. N. (1981) *Biochemistry, 20*, 5025–5032; Konings, W. N. and Robillard, G. T. (1982) *Proc. Natl Acad. Sci. USA*, in the press]. Based on the similarities between the data from these three systems and published data from other systems, we now propose that dithiol-disulfide interchange may play a general role in membrane-related processes such as transport, energy transduction and hormone-receptor interactions.

We propose that the affinities of the substrate-binding sites are regulated by a dithiol and a disulfide situated at different depths in the membrane. In addition we propose that the oxidation states of these two redox centers are coupled by dithiol-disulfide interchange such that, when one is oxidized, the other is reduced. Since a transmembrane electrical potential, $\Delta\psi$, or a pH gradient, ΔpH, can alter the redox state, it can change the affinity of the substrate-binding sites. The $\Delta\tilde{\mu}_{H^+}$-induced changes in affinity are sufficient to drive active transport (symport or antiport) and energy-transducing processes. A similar mechanism can be applied to transport systems driven by phosphorylated enzyme intermediates instead of $\Delta\tilde{\mu}_{H^+}$.

Changes of the redox potential in a given compartment during metabolism could also control the affinity of ligand binding even in the absence of a $\Delta\tilde{\mu}_{H^+}$. The ligand-binding affinities of facilitated diffusion transport systems and receptor proteins may be regulated in this manner.

The number of reports citing the involvement of sulfhydryl groups in the function of membrane-bound proteins such as solute-transport proteins, energy-transducing enzymes or receptor proteins has become so large that it is time to consider whether sulfhydryl groups play a general role in membrane-related processes.

The activity of the phospho*enol*pyruvate-dependent hexose transport system in *Escherichia coli* can be inhibited by sulfhydryl reagents [1]. It can also be inhibited by a $\Delta\tilde{\mu}_{H^+}$ [2,3]. We have recently shown that the inhibition by $\Delta\tilde{\mu}_{H^+}$ arises through an oxidation of a dithiol to a disulfide which changes the membrane-bound transport enzyme from a high affinity to a low affinity form. An identical conversion, in the absence of a $\Delta\tilde{\mu}_{H^+}$ could be achieved by changing the redox potential of the membrane with oxidizing or reducing agents [4]. Certain similarities between the phosphotransferase system and $\Delta\tilde{\mu}_{H^+}$-driven transport systems relative to their response to sulfhydryl reagents and the influence of $\Delta\tilde{\mu}_{H^+}$ on K_m [5] stimulated a more recent investigation of lactose and proline transport in *E. coli*. We have demonstrated that the K_m can be altered to the same extent either by changing the redox potential in the membrane or by (de)energizing the membrane. The redox-sensitive step is again a dithiol-disulfide interconversion [6].

Dithiol-disulfide conversions have been reported to play a role in such diverse systems as the Ca^{2+} [7] and P_i [8] transport systems and the NADH dehydrogenase [9] of mitochondria, the γ-aminobutyric acid transport system in brain synaptosomes [10], the H^+-ATPase in chloroplasts [11–15], the acetylcholine receptor in *Torpedo californica* electroplax membranes [16], the myosin subfragment one from rabbit skeletal muscle [17,18] and the glucose-transport systems from thymocytes and adipocytes [19,20]. Such studies, together with reports that the sensitivity of various transport proteins to sulfhydryl reagents is influenced either by the addition of substrates or energization of the membrane or both (see [21] for a review), suggest that, in those systems where sulfhydryl groups are functional, they may fulfill a general role. The purpose of this work is to bring together some commonly recurring features of –SH group reactivity and changing K_m values in various membrane-bound systems into a framework which is amenable to experimental tests.

Abbreviation. MalNEt, *N*-ethylmaleimide.

Note. K_m can be equated either with a real dissociation constant K_d or with K_T, the half saturation constant for transport, which includes both the binding constant and the rate constant for the transport step. Since there is no definitive evidence that the K_m is not equal to K_d in the systems discussed here we shall speak of changes in K_m and being indicative of changes in affinity states.

RESULTS

Relationship between Redox Potential and Transmembrane Electrical Potential

Divide a biological membrane into two sectors, the outer and inner sector and let each sector contain an identical but immobile redox center (see Fig. 1). In solution the midpoint potentials of these two centers are equal. However, when

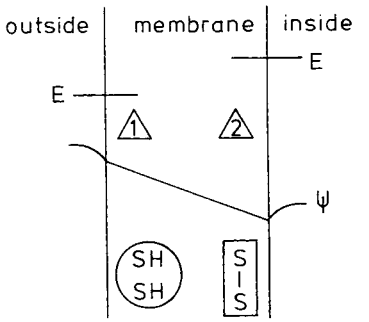

Fig. 1. *Relationship between $\Delta\psi$ and the redox potential in the membrane.* ψ is the electrical potential and E is the redox potential

situated in different sectors of a membrane their redox states will vary in response to a transmembrane electrical potential. This can be seen from the following considerations.

The electrochemical potential of an electron in redox center 1 is

$$\tilde{\mu}_e^1 = -FE^0 + \frac{RT}{n} \ln \frac{a_R^1}{a_O^1} - F\psi^1. \quad (1)$$

Rewriting Eqn (1) in terms of the redox potential

$$E = E^0 + \frac{RT}{Fn} \ln \frac{a_O}{a_R}$$

gives

$$\tilde{\mu}_e^1 = -F(E^1 + \psi^1). \quad (2)$$

A similar expression can be written for the electrochemical potential of the electron in redox center 2. If mobile electron carriers can interact with each redox center or if reducing equivalents can be transferred between centers along special pathways, the electrochemical potential of the electron at both centers will be in equilibrium ($\tilde{\mu}_e^1 = \tilde{\mu}_e^2$) even when an electrical potential is being maintained across the membrane by proton pumps. As shown in Fig. 1, the electrical potential, ψ, at redox sites 1 and 2 is no longer equal under these circumstances. As a result, the redox potential at each site will shift to compensate for the difference in electrical potential while maintaining equilibrium for the electrochemical potential of the electron at both sites (see [22] for a detailed treatment)

$$-F(E^1 + \psi^1) = \tilde{\mu}_e^1 = \tilde{\mu}_e^2 = -F(E^2 + \psi^2) \quad (3)$$

$$\Delta\psi = \psi^2 - \psi^1 = E^1 - E^2. \quad (4)$$

Consequently, when the electrical potential at site 2 is lower than at site 1, as in the case when $\Delta\psi$ is interior-negative, the redox potential at site 2 will be higher than at site 1. This is illustrated in the lower part of Fig. 1 using a dithiol as the oxidizable moiety at each site. Reversing the polarity of the electrical potential reverses the difference in redox potential. In 1970 Hinkle and Mitchell experimentally demonstrated that establishing a $\Delta\psi$ across the membrane altered the redox state of components in the membrane [23].

Relationship between the Redox State and ΔpH

The oxidation state of each of the membrane bound redox centers in Fig. 1 can be altered by a change in pH if the centers respond directly to solution pH or if they respond indirectly via changes in the pK of neighboring ionizable groups. The pH dependence of the redox potential for each redox center can be written as

$$E = E^0 + \frac{RT}{nF} \ln \frac{[ox]}{[red]} + \frac{RT}{nF} \ln \frac{(1 + a_{H^+}/K_O)}{(1 + a_{H^+}/K_R)} \quad (5)$$

if the redox couple binds only one proton. K_R and K_O are the equilibrium protonation constants for the reduced and oxidized species respectively. The pK of the reduced species $RSH \longleftrightarrow RS^- + H^+$ should be in the range of 8–10 while that of the oxidized species $RSH^+ \longleftrightarrow RS^{\cdot} + H^+$ should be considerably lower. Consequently the reduced species will be protonated and the oxidixed species deprotonated at physiological pH and Eqn (5) simplifies to

$$E = E^0 + \frac{RT}{nF} \ln \frac{[ox]}{[red]} - \frac{RT}{nF} \ln (1 + a_{H^+}/K_R). \quad (6)$$

Since $E = E_m$ when [ox]/[red] = 1, Eqn (6), written in terms of the midpoint potential, becomes

$$E_m = E + \frac{2.3\,RT}{nF} pK_R - \frac{2.3\,RT}{nF} pH. \quad (7)$$

Eqn (7) predicts that a decrease in the solution pH or an increase in the pK of the reduced redox species shifts the equilibrium towards the reduced state. Such is the pH dependence normally observed for the oxidation of thiols.

Using this pH or pK dependence and replacing $\Delta\psi$, interior-negative, in Fig. 1 by a ΔpH, interior-alkaline, we see that the ΔpH-induced changes in redox state at each site are analogous to the $\Delta\psi$-induced changes. The outer site becomes more reduced and the inner site more oxidized relative to the oxidation states of these sites in the absence of the ΔpH.

Arrangement of Redox Centers

When α-methylglucose phosphorylation activity was measured using inverted *Escherichia coli* cytoplasmic membrane vesicles (reactants and products are on the right side of the membrane in Fig. 1) we showed that addition of oxidizing agents caused approximately a 500-fold increase in K_m due to the oxidation of a dithiol [4]. An identical change in K_m was effected by establishing a $\Delta\tilde{\mu}_{H^+}$ of the same polarity as in Fig. 1. Either oxidation or the $\Delta\tilde{\mu}_{H^+}$ protected the carrier from inhibition by sulfhydryl reagents including non-permeant reagents such as glutathione hexane maleimide. This response to the $\Delta\tilde{\mu}_{H^+}$ clearly reflects the redox behavior predicted for a dithiol situated in region 2 of the membrane. On the other hand when α-methylglucose transport activity was measured using intact spheroplasts or cytoplasmic membrane vesicles we observed that a $\Delta\mu_{H^+}$ of the same polarity as shown in Fig. 1 stimulated inhibition by MalNEt and glutathione hexane maleimide (unpublished results). In this case the reagents were added to the left side of the membrane in Fig. 1. This is the predicted response towards a $\Delta\mu_{H^+}$, interior negative and alkaline, if a dithiol is located in region 1 of the membrane. Thus there are dithiols on both sides of the membrane associated with carrier activity. These data are consistent with the concept of a movable carrier which can shift one set of dithiols between regions of differing redox potential by rotating or shuttling back and forth in the membrane. But the data are also consistent with the concept of two sets of dithiols on a non-movable carrier or channel where one set is located near the outer surface and the other near the inner surface. In the remainder of this presentation we will

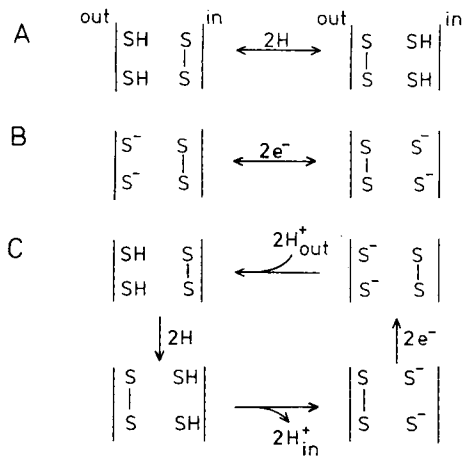

Fig. 2. *Proposed pathways of dithiol-disulfide interchange*

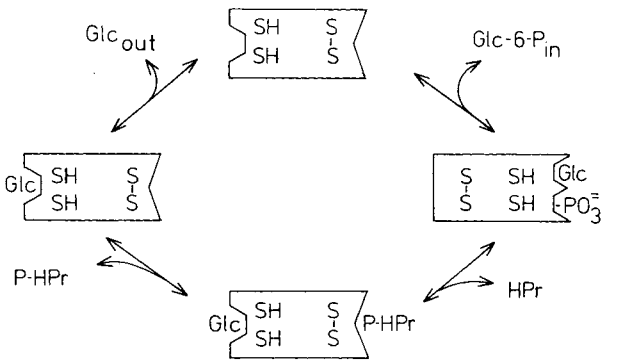

Fig. 3. *Proposal for a redox-coupled transport by phosphorylated enzyme intermediates*

treat carriers as channels with fixed redox centers at both ends. Nevertheless, the same concepts can be readily adapted to a movable carrier with one set of dithiols.

When a single dithiol is the only redox element in the enzyme it will have to interact with exogenous electron acceptors such as ubiquinone in order to change it's oxidation state. Monitoring the effect of respiratory-chain inhibitors on the redox state of the dithiol should yield data concerning this type of coupling. However, when there are two sets of interacting dithiols, the need for exogenous electron donors/acceptors ceases because one redox center can now function as the donor/acceptor for the other. The mechanisms for passing reducing equivalents back and forth between two fixed sites are presented in Fig. 2. Hydrogen can be moved as in Fig. 2 A or electrons as in Fig. 2 B where no net translocation of H or e^- occurs in a redox cycle which takes the outer site from reduced to oxidized and back to reduced. But the coupling could also take place by a mechanism which moves hydrogen in one direction and electrons in the reverse direction as shown in Fig. 2 C. This last mechanism results in the net translocation of protons during a redox cycle. If the two sets of dithiols are not adjacent, movement of hydrogen could be achieved by a series of adjacent sulfhydryl groups or, possibly, hydrogen-bonded side chains while the pathway for moving electrons could involve prosthetic groups, metals or tunnelling via aromatic side chains.

When the redox states of these centers control the affinity states of substrate-binding sites, the change in redox state can be incorporated into the mechanism of transport and transducing enzymes in various ways as will be presented in the following sections. The general model which we will use is that the carrier protein or protein complex forms a channel through the membrane. At each end of the channel there is a redox center and the oxidation state of these two centers are coupled.

Transport Driven by Phosphorylated Enzymes

Measurements of glucose phosphorylation activity [24] have demonstrated that the glucose transport enzyme, E_{II}, cycles through a high-affinity and low-affinity form for glucose during the sequence in Scheme1.

$$P\text{-HPr} + E_{II} \longleftrightarrow \text{HPr} + P\text{-}E_{II}$$

$$P\text{-}E_{II} + \text{Glc}_{out} \longleftrightarrow E_{II} + \text{Glc-6-}P_{in}.$$

Scheme 1

E_{II} is high affinity for *P*-HPr and low affinity for glucose while *P*-E_{II} is low affinity for *P*-HPr and high affinity for glucose. The redox mechanism which we have shown to be operative could account for these affinity changes as shown in Fig. 3. Here the phosphoryl group brings about the shift in redox states. Binding a negatively charged phosphoryl group close to the dithiol will raise the apparent p*K* and consequently the midpoint potential of the dithiol-disulfide transition. At a constant redox potential, therefore, phosphorylation of E_{II} at the inner site could shift this site from the oxidized to the reduced form. Since the reduced form is the high-affinity form, glucose binds first at the outer site. After phosphorylation shifts the inner site to the high-affinity form, glucose is released from the outer site, drawn through the channel and bound at the inner site. When the phosphoryl group is shifted to glucose the dephosphorylated enzyme shifts back to the oxidized form releasing glucose 6-phosphate and completing the cycle.

A similar mechanism may be applicable to other transport systems driven by phosphorylated enzyme intermediates. An obvious example is the sarcoplasmic reticulum Ca^{2+}-ATPase [25]. It possesses Ca^{2+}-binding sites on the inner and outer side of the membrane. Normally the outer site is high affinity and the inner site low affinity with K_d values of 0.5–2 μM and 0.5–1 mM, respectively. The outer site converts from high to low affinity during ATP hydrolysis. Conformational changes as a result of Ca^{2+} and ATP binding have been detected by physical probes and changes in the –SH group reactivity due to Ca^{2+} plus ATP binding are known to occur. The system differs from the phosphotransferase system, however, in that ATP phosphorylates from the outer side. Ikemoto [26] has shown that Ca^{2+} is released when the ATPase is phosphorylated. Relative to the scheme in Fig. 3, Ikemoto's results would suggest that once Ca^{2+} is bound to the inner high-affinity site, phosphorylation at the outer oxidized site would shift the inner site to the oxidized low-affinity form, stimulating the release of Ca^{2+} to the interior.

$\Delta\tilde{\mu}_{H^+}$-Driven Solute Transport

Many active transport carriers convert between forms with high and low K_m values. K_m values for $\Delta\tilde{\mu}_{H^+}$-driven transport of lactose are a factor of 100 lower than the K_m for facilitated diffusion [5]. We demonstrated that lipophilic oxidizing agents are capable of inhibiting lactose and proline efflux and counter-

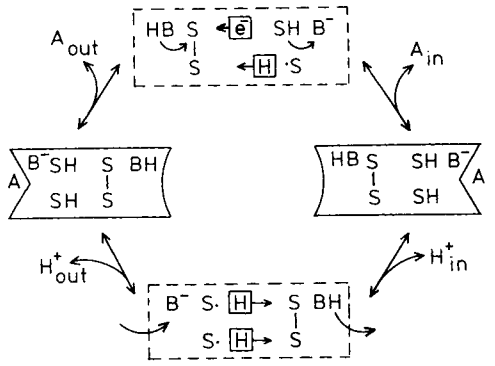

Fig. 4. *Proposal for a redox-controlled H^+/solute symport.* A is the solute being transported

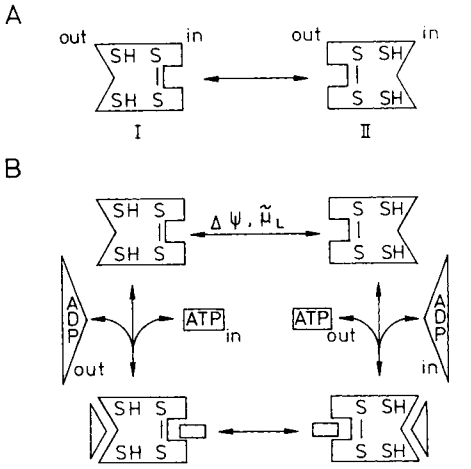

Fig. 5. *Proposal for a redox and $\Delta\tilde{\mu}_{H^+}$ influenced facilitated diffusion*

flow as well as uptake driven by ascorbate/phenazine methosulfate oxidation or an artificially generated membrane potential. The inhibition is reversed by reducing agents [6]. Oxidizing agents also protect the carrier from inactivation by MalNEt. The K_m of the oxidized and reduced carrier are the same as the K_m of the non-energized and energized carrier, respectively. These data prove that dithiol-disulfide conversions play a role in determining the K_m of the carrier. It has also been shown recently that inactivation of lactose and proline transport systems in membrane vesicles by glutathione hexanemaleimide is stimulated by a $\Delta\tilde{\mu}_{H^+}$ of the same polarity as shown in Fig. 1 [27]. The response of these systems is analogous to that discussed in the preceding section, but the driving force is different. Fig. 4 combines the general concept used in Fig. 2 with $\Delta\tilde{\mu}_{H^+}$ as a driving force.

H^+ Symport

Upon energization, $\Delta\psi$, interior-negative, or ΔpH, interior-alkaline, shifts the equilibrium towards the state in which all the outer sites are reduced high-affinity, and the inner sites are oxidized low-affinity. A cycle of substrate transport in symport with protons can proceed as shown in Fig. 4. Ligand binds at the outer high-affinity site. Binding of protons on the base, B^-, in the vicinity of the dithiol will cause a decrease both in the apparent pK of the dithiol and the midpoint potential of the redox transition resulting in the oxidation of this center. Hydrogens move across the membrane reducing the inner redox center and raising the pK of the inner base, BH. Consequently the base deprotonates releasing protons to the inner solution. The change in redox states of the inner and outer sites causes the outer site to become low affinity and the inner site high affinity. The high local concentration of substrate at the outer site now in its low-affinity conformation, raises the probability that it can be drawn through the channel and bound at the inner site now in its high-affinity conformation. At this point protons and solute have been translocated but the carrier is out of redox equilibrium. This can be re-established by movement of electrons back to the outer redox center. The inner site becomes oxidized and releases substrate and the outer site becomes reduced to complete the cycle. In order to avoid having created a leak for H^+ by such a mechanism, we must include the restriction that H^+ be translocated only if the solute is transported.

The expected behavior of the system predicted from the mechanism in Fig. 4 is in keeping with the observations made on lactose efflux and exchange by Kaczorowski et al. [5,28]. Efflux is the backward reaction with concomitant proton and ligand movement. The higher internal ligand potential induces the inner site into the high-affinity, reduced form. Ligand flows to the exterior resulting in proton pumping [28,29]. ΔpH or $\Delta\psi$, interior alkaline or negative, should decrease the rate of efflux since they generate a proton flow opposing that accompanying ligand efflux. Consequently, they will inhibit ligand-driven isomerization of the carrier. In contrast to efflux, exchange should not be influenced by ΔpH or $\Delta\psi$. The carrier in Fig. 4 is symmetric. If the ligand concentration on both sides of the membrane is the same, an inhibitory effect on efflux will be countered by a stimulatory effect on influx. Analogous reasoning applies for $\Delta\psi$, interior positive as well as ΔpH, interior acid or alkaline.

H^+ Antiport

The same model postulated in Fig. 4 for H^+ symport can also be used for H^+ antiport. In such a system the oxidized form would be high affinity and the reduced form low affinity.

$\Delta\tilde{\mu}_{H^+}$-Influenced Facilitated Diffusion

The mitochondrial adenine nucleotide translocator carries one ADP into the mitochondria in exchange for one ATP carried out. Both ligands flow down their respective concentration gradients. In the non-energized mitochondria, the translocator shows little selectivity for ADP over ATP. Upon energization, however, the K_m for ADP_{ext} decreases and that for ATP_{ext} increases for a total change of approximately 150 between the two K_m values. The translocator activity is sensitive to sulfhydryl reagents, the sensitivity being a function of the state of energization of the membrane as well as the presence of substrate. In addition, oxidizing agents inhibit translocator activity. A mechanism analogous to that in Fig. 4 can explain these observations.

In its simplest version (Fig. 5 A) the translocator possesses two sites, one on either side of the membrane. ADP and ATP can bind to both sites. The site in its reduced form is high affinity for ADP and low affinity for ATP. In the oxidized form the reverse is true. In the non-energized membrane the two ligands are competitive because each ligand induces the translocator into its own high affinity form which is, by definition, low affinity for the corresponding ligand. Upon

establishing a $\Delta\tilde{\mu}_{H^+}$, interior negative and alkaline the equilibrium is shifted towards state I and the translocator becomes asymmetric relative to its binding characteristics (Fig. 5A). All outer sites become high affinity for ADP and low affinity for ATP; the reverse holds for the inner sites. Translocation would proceed as illustrated in Fig. 5B. ADP binds preferentially to the external site and ATP to the internal site. Isomerization of the carrier follows, driven by the high chemical potential of ADP_{ext} and ATP_{int}. ADP and ATP translocate and bind to their new high-affinity sites. The high chemical potential of ADP_{ext} and ATP_{int} shift the carrier back to state I and the ligands are released. Reversing the polarity of $\Delta\tilde{\mu}_{H^+}$ reverses the specificity resulting in the movement of ATP into and ADP out of the mitochondria. This reversibility has been demonstrated using the purified translocator reconstituted into proteoliposomes [30].

A number of important experimental observations are consistent with this proposal. The inhibitor bongkrekate binds to the translocator on the matrix side of the membrane and increases the number of available ADP-binding sites. ADP_{ext} enhances, to approximately the same extent, the number of bongkrekate-binding sites on the matrix site [31,32]. Thus, bongkrekate most likely binds to the inner site in its oxidized form, shifting the equilibrium towards state I, and increasing the number of ADP sites. External ADP effects the number of bongkrekate-binding sites in an analogous manner. The observation of a ternary bongkrekate :ADP:translocator complex and the kinetic evidence for a ternary ANP:ANP:translocator complex are consistent with such a model [33,34]. That bongkrekate and carboxyatractyloside binding are mutually exclusive [35] suggests that carboxyatractyloside binds to the outer side in its oxidized form, shifting translocator to state II and inhibiting bongkrekate binding.

Energization of the membrane has been reported to unmask a considerable number of translocator $-SH$ groups which can then react with MalNEt [36] resulting in inhibition of translocator activity and reduced binding of [^{35}S]atractyloside [37]. These results are expected. During short exposure to MalNEt reaction at the internal sites will be retarded by prerequisite diffusion of MalNEt through the membrane. Energization shifts the translocator to state I with the outer sites in their reduced form leading to increased reaction with MalNEt. Since atractyloside normally binds to the outer sites, decreased binding should be expected. At least some of these essential sulfhydryl groups appear to be juxtaposed and capable of undergoing oxidation to disulfides. This is evident from experiments showing that triethyl and tributyl tin salts and anthraquinone derivatives inhibit translocator activity [38,39].

Finally, since energization catalyses the isomerization back to state I, it is easy to see that it may also influence the velocity of the exchange reaction as well as the affinities of the individual sites.

Energy Transduction and Proton Pumping

Some elements of Fig. 2 and 4 can be applied to energy transduction using the mitochondrial pyridine nucleotide transhydrogenase system to demonstrate this concept. The same proposal is applicable to H^+-ATPase as will be discussed later.

The transhydrogenase catalyzes the reaction $NADP^+ + NADH \longleftrightarrow NADPH + NAD^+$. In the absence of $\Delta\tilde{\mu}_{H^+}$ approximately equal concentrations of substrates and products

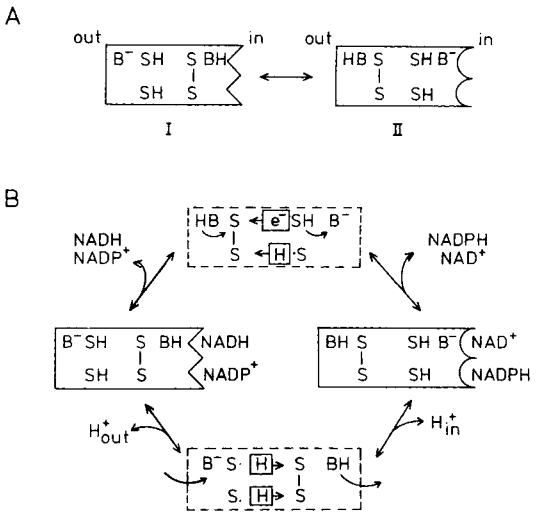

Fig. 6. *Proposal for a redox-coupled energy transducing system*

are reached at equilibrium (non-energized reaction). In the presence of $\Delta\tilde{\mu}_{H^+}$, however, the equilibrium of the reaction is shifted far to the side of NADPH and NAD^+ at the expense of proton translocation across the membrane (energized reaction).

It has been shown that the strength of substrate binding is influenced by the state of energization of the membrane [40] and it has also been shown that NADPH-binding sensitizes one set of sulfhydryl groups to MalNEt reaction, while $NADP^+$ binding protects against MalNEt inactivation [41]. In addition, compounds which can function as oxidants, dibromothymoquinone [42] 2-phenylindolone [43] and Cd^{2+}, [9] inhibit the enzyme.

These elements are consistent with a transhydrogenase which possesses one set of sites for binding either $NADP^+$ and NADH or NADPH and NAD^+. The sites can isomerize between high and low affinity states and this isomerization determines which set of substrates is bound.

Energy-Linked Reaction. Establishment of $\Delta\psi$ and/or ΔpH interior negative and/or alkaline shifts the equilibrium in Fig. 6A to state I, the same redox configuration as seen in the previous systems. The $\Delta\mu_{H^+}$-driven NADPH production occurs as follows. $NADP^+$ and NADH preferentially bind to the conformation in state I. After binding of these substrates at their high-affinity binding sites, H^+ binding at the outer dithiol forces a shift in redox states bringing the enzyme:$NADP^+$:NADH complex into a conformationally altered state which induces the formation of NADPH and NAD^+. The conformationally altered state (state II) containing bound $NADP^+$ and NADH can be viewed as the energized state. Conversion of $NADP^+$ and NADH to NADPH and NAD^+ requires no further energy input. Once formed the tightly bound NADPH and NAD^+ will be released when redox equilibrium is re-established by passage of electrons back to the outer disulfide, converting the binding site back to a low-affinity configuration for NADPH and NAD^+. By cycling in this manner, high concentrations of NADPH can be generated at the expense of the proton concentration gradient. This proposal provides a mechanism for the types of energy coupling suggested by Boyer [44,45].

Non-Energized Reaction. NADPH and NAD^+ bind more strongly to the non-energized form (state II) than to the energized form (state I) [40]. Beginning with excess NADPH

and NAD^+ the reverse transhydrogenase reaction proceeds in a clockwise direction with binding of the substrates. After hydride transfer the newly formed NADH and $NADP^+$, which have a higher affinity for state I, will induce isomerization. This will be accompanied by proton binding on the matrix side, dithiol-disulfide interchange and release of a proton into the cytosol. The reaction will continue to cycle in a clockwise sequence faster than in a counter clockwise sequence until equilibrium has been reached. The net result will be the pumping of protons to the exterior. The forward reaction, beginning with excess $NADP^+$ and NADH, cycles in a counterclockwise manner resulting in proton pumping to the interior.

H^+-ATPase. Boyer has proposed that proton-induced changes in the ATPase conformation near the active site could result in ATP synthesis [44,45]. Our proposal in the previous paragraphs shows how this coupling could occur. Conformational changes in chloroplast and mitochondrial H^+-ATPases in response to membrane energization are well documented [46–51] as are changes in the K_m and sulfhydryl group reactivity of the ATPases upon energization [52,53]. The catalytic activity of the heat-activated or light-activated ATPase is inhibited by MalNEt, indicating that conformational changes occur during the activation in which sulfhydryl groups become available [54–56], Farron and Racker [55] proposed that the conformational change which occurred upon heat activation proceeded via sulfhydryl-disulfide interchange. Recently Ravizzini et al. [11] have confirmed this proposal by determining the distribution of disulfide and sulfhydryl groups in CF_1. The complex contained eight free sulfhydryl groups distributed in the ratio 4:2:0:0:2 on the α, β, γ, δ and ε subunits respectively. Expression of the ATPase activity by heat activation was accompanied by a sulfhydryl-disulfide interchange between the α and γ subunits resulting in a new sulfhydryl group distribution of 2:2:2:0:2. Two sulfhydryl groups on one α subunit become oxidized to a disulfide while one disulfide on the γ subunit is reduced to the sulfhydryl form. The sulfhydryl-disulfide interchange in the γ subunit appears to influence the conformational equilibria which regulate the CF_1 activity. Modification with various sulfhydryl reagents influences the activity in different ways depending on the type of reagent and the extent of modification. Upon illumination of chloroplast thylakoids with MalNEt, the γ subunit of CF_1 was specifically labeled concomitant with a partial inhibition of photophosphorylation. Light-induced H^+ uptake was not inhibited but ATP stimulated H^+ uptake decreased by 70% [57,58]. *o*-Phenylenedimaleimide reacted with chloroplast thylakoids during illumination, causing inhibition of photophosphorylation and light-dependent proton uptake as well as increased proton permeability of the system. Labeling studies suggested that crosslinking occurs between two sulfhydryl groups on the γ subunit, one which is available in the dark and one which becomes available upon light activation [12]. Photophosphorylation was inhibited by 2-2′-dithiobis-5-nitropyridine in spinach chloroplasts when the reagent was present during illumination. The appearance of two moles of thione for each mole of reagent used was taken as evidence for the reformation of a disulfide from two vicinal sulfhydryls [59,60]. Illumination of chloroplasts in the presence of Cu^{2+} also results in inhibition of photophosphorylation. The inhibitory effects of Cu^{2+} and MalNEt are additive. Prior treatment with Cu^{2+} reduced the level of light-induced MalNEt labeling in the γ subunit [61]. These data also support the concept of disulfide-bond formation within the γ subunit accompanying inhibition of photophosphorylation. Andreo et al. [62] demonstrated that reaction of purified CF_1 with iodosobenzoate led to partial inhibition of the Ca^{2+}-ATPase activity. Two free sulfhydryl groups in the γ subunit and two in the β subunit were oxidized to disulfides. The same iodosobenzoate treatment applied to spinach chloroplast thylakoid membranes inhibited both photophosphorylation and Mg^{2+}-ATPase activity and resulted in the formation of the same two disulfides in the γ and β subunits [59,60,62]. Dithiols also appear to have a function in gating the F_0 channel. The oxidant, triphenyl tin, inhibits proton translocation through the channel and dithiothreitol restores the translocation activity [13,14].

The occurrence of dithiol-disulfide interchange in the F_0 channel, the γ subunit which gates the channel and the catalytic and regulatory subunits suggest that dithiol-disulfide interchange may play a functional role in ATP synthesis. Experimental support for such a proposal may be found in the recent work of Vinkler and Kornstein [63]. They demonstrated that an externally applied electric field could drive ATP synthesis in a chloroplast suspension and that this reaction was not dependent on transmembrane ion gradients or proton translocation since ionophores and *N,N′*-dicyclohexylcarbodiimide did not inhibit the process. ATP synthesis increased linearly with the number of pulses and the pulse width, eventually saturating at large pulse widths, and it continued once the field was turned off. This led the authors to conclude that there was an accumulation of some intermediate which drove ATP synthesis. We suggest that the intermediate could be electrons. The membrane can be compared with a capacitor which becomes charged upon exposure to the electric field and then slowly discharges by passing its electrons through the various redox centers in the membrane, including those of the ATPase, on the way to solution. If the redox states of these centers control the affinities of ADP and ATP binding then passage of electrons through these centers could generate the affinity changes required for ATP synthesis according to the conformational model. At the present time there is no data available concerning redox control of ADP and ATP binding affinities.

Concluding Remarks

The mechanism presented above consists of only two essential features.

1. Ligand binding sites whose affinities are regulated by the redox state of vicinal sulfhydryl groups. Using two sets of vicinal sulfhydryls with coupled redox states keeps the electron donor and acceptor within one complex where the bulk of the protein can insulate it from changes in the redox state of other centers in the membrane. If we remove this constraint, one set of vicinal sulfhydryls would be sufficient to regulate the affinity of one or more binding sites provided that some other redox mediator is available to serve as electron donor or acceptor when necessary.

2. Alternating affinities of the sites mediated by dithiol-disulfide interchange. The advantage of using two sites with alternating affinites for transport is that it explains how the binding sites can be accessible from both sides on the membrane without having to invoke rotation of the proteins from one side to the other. Since the affinities of the sites on both sides can be altered by energization, transport can be driven in either direction depending on the polarity of $\Delta\tilde{\mu}_{H^+}$. As stated earlier, however, one binding site controlled by one or two dithiols is sufficient on a rotating carrier. All other features

of the mechanism including the number of H^+ translocated per cycle are flexible. Whether the dithiol-disulfide interchange is direct or makes use of channels for hydrogen and electron transfer is flexible, as is the nature and number of the proton-transferring groups, BH, between solution and the redox site. The location and accessibility of the vicinal sulfhydryl group will also vary with the system. Such variation has been observed. Some systems are inactivated by penetrant (apolar) and non-penetrant (polar) reagents while others are inactivated only by penetrant reagents. These differences have led to the conclusion that the reactive sulfhydryls are accessible from the outer side of the compartment in some systems and from the inner side in others [21]. However, when careful studies have been done with both types of reagents [8], or with a single reagent and membranes with a normal and inverted orientation [64], these correlations break down. It is more likely that the accessibility at each site is controlled by local structure, for example, proximity to the surface, hydrophobicity of the immediate surroundings and steric hindrances. This feature will vary for each system.

Gating Effects. Gating effects have been observed in $\Delta\tilde{\mu}_{H^+}$-driven processes such as the Na^+/H^+ antiporter in vesicles of *Halobacterium halobium* and H^+-ATPases. These observations are readily explainable when the activity of a system is coupled to a redox conversion. A finite $\Delta\psi$, the magnitude of which will be governed by conditions such as ionic strength, pH and temperature, will be necessary to elevate the redox potential past the midpoint potential of a given site before oxidation can occur.

Non-energized Membranes. The emphasis in this treatment has been placed on systems which are found in energizable membranes. As shown in Fig. 1 the normal polarity of the $\Delta\tilde{\mu}_{H^+}$ determines that the reduced form will be high affinity for substrates being transported into the cell. In non-energized membranes no such constraint is necessary and one might expect to find certain systems in which the oxidized form is the more relevant species. Glucose transport in adipocytes is the best example [65].

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REFERENCES

1. Haguenauer-Tsapis, R. & Kepes, A. (1977) *Biochim. Biophys. Acta, 465*, 118–130.
2. Hernandez-Asensio, M., Ramirez, J. M. & del Campo, F. F. (1975) *Arch. Microbiol. 103*, 155–162.
3. Reider, E., Wagner, E. F. & Schweiger, M. (1979) *Proc. Natl Acad. Sci. USA, 76*, 5529–5533.
4. Robillard, G. T. & Konings, W. N. (1981) *Biochemistry, 20*, 5025–5032.
5. Kaczorowski, G. J., Robertson, D. E. & Kaback, H. R. (1979) *Biochemistry, 18*, 3697–3704.
6. Konings, W. N. & Robillard, G. T. (1982) *Proc. Natl Acad. Sci. USA*, in the press.
7. Rugolo, M., Siliprandi, D., Siliprandi, N., Toninello, A. & Zoccarato, F. (1981) *Biochem. Soc. Trans. 9*, 135P.
8. Fonyo, A. & Vignais, P. V. (1980) *J. Bioenerg. Biomembr. 12*, 137–149.
9. Kaufman, B. & Kaplan, N. O. (1961) *J. Biol. Chem. 236*, 2133–2139.
10. Kanner, B. I. (1978) *Biochemistry, 17*, 1207–1211.
11. Ravizzini, R. A., Andreo, C. S. & Vallejos, R. H. (1980) *Biochim. Biophys. Acta, 591*, 135–141.
12. Weiss, M. A. & McCarty, R. E. (1977) *J. Biol. Chem. 252*, 8007–8012.
13. Gould, J. M. (1976) *Eur. J. Biochem. 62*, 567–575.
14. Gould, J. M. (1978) *FEBS Lett. 94*, 90–94.
15. Sone, N., Yoshida, M., Hirata, H. & Kagawa, Y. (1975) *J. Biol. Chem. 250*, 7917–7923.
16. Walker, J. W., Lukas, R. J. & McNamee, M. G. (1981) *Biochemistry, 20*, 2191–2199.
17. Wells, J. A., Werber, M. A. & Yount, R. G. (1979) *Biochemistry, 18*, 4800–4805.
18. Wells, J. A. & Yount, R. G. (1980) *Biochemistry, 19*, 1711–1717.
19. Regen, D. M., Schraw, W. P., Tarpley, H. L. & Juliao, S. F. (1981) *Biochim. Biophys. Acta, 644*, 62–68.
20. Czech, M. P. (1976) *J. Biol. Chem. 251*, 1164–1170.
21. Fonyo, A. (1978) *J. Bioenerg. Biomembr. 10*, 171–194.
22. Walz, D. (1979) *Biochim. Biophys. Acta, 505*, 279–353.
23. Hinkle, P. & Mitchell, P. (1970) *Bioenergetics, 1*, 45–60.
24. Misset, O. & Robillard, G. T. (1982) *Biochemistry, 21*, 3136–3142.
25. De Meis, L. & Vianna, A. L. (1979) *Annu. Rev. Biochem. 48*, 275–292.
26. Ikemoto, N. (1976) *J. Biol. Chem. 251*, 7275–7277.
27. Cohn, D. E., Kaczorowski, G. T. & Kaback, H. R. (1981) *Biochemistry, 20*, 3308–3313.
28. Kaczorowski, G. J. & Kaback, H. R. (1979) *Biochemistry, 18*, 3691–3696.
29. Otto, R., Sonnenberg, A. S. M., Veldkamp, H. & Konings, W. N. (1980) *Proc. Natl Acad. Sci. USA, 77*, 5502–5506.
30. Kramer, R. & Klingenberg, M. (1980) *Biochemistry, 19*, 556–560.
31. Klingenberg, M. & Buchholz, M. (1973) *Eur. J. Biochem. 38*, 346–358.
32. Erdelt, H., Weidemann, M. J., Buchholz, M. & Klingenberg, M. (1972) *Eur. J. Biochem. 30*, 107–122.
33. Lauquin, G. J. M. & Vignais, P. V. (1976) *Biochemistry, 15*, 2316–2327.
34. Barbour, R. L. & Chan, S. H. P. (1981) *J. Biol. Chem. 256*, 1940–1948.
35. Vignais, P. V., Vignais, P. M. & Defaye, G. (1973) *Biochemistry, 12*, 1508–1519.
36. LeQuoc, K., LeQuoc, D. & Gaudemer, Y. (1979) *Biochim. Biophys. Acta, 546*, 356–364.
37. Vignais, P. V. & Vignais, P. M. (1972) *FEBS Lett. 26*, 27–31.
38. Harris, E. J., Bangham, J. A. & Zukovic, B. (1973) *FEBS Lett. 29*, 339–344.
39. Boos, K. S. & Schlimme, E. (1981) *FEBS Lett. 127*, 40–44.
40. Rydstrom, L., Teixeira Da Cruz, A. & Ernster, L. (1971) *Eur. J. Biochem. 23*, 212–219.
41. Earle, S. R., O'Neal, S. G. & Fisher, R. R. (1978) *Biochemistry, 17*, 4638–4690.
42. Houghton, R. L., Fisher, R. J. & Rao Sanadi, D. (1976) *FEBS Lett. 68*, 95–98.
43. Green, A. P., Sweetman, A. J., Hooper, M., Cattell, K. J., Lindop, C. R. & Beechy, R. B. (1973) *Biochem. Soc. Trans. 1*, 410–412.
44. Boyer, P. D. (1975) *FEBS Lett. 58*, 1–4.
45. Boyer, P. D. (1977) *Annu. Rev. Biochem. 46*, 957–966.
46. Yeates, R. A. (1974) *Biochim. Biophys. Acta, 333*, 173–179.
47. Bertina, R. M., Schrier, P. E., & Slater, E. C. (1973) *Biochim. Biophys. Acta, 305*, 503–518.
48. Chang, T. M. & Penefsky, H. S. (1973) *J. Biol. Chem. 248*, 2746–2754.
49. Chang, T. M. & Penefsky, H. S. (1974) *J. Biol. Chem. 249*, 1090–1098.
50. Ryrie, I. J. & Jagendorf, A. T. (1971) *J. Biol. Chem. 246*, 3771–3774.
51. Ryrie, I. J. & Jagendorf, A. T. (1972) *J. Biol. Chem. 247*, 4453–4459.
52. Kayalar, C., Rosing, J. & Boyer, P. D. (1976) *Biochem. Biophys. Res. Commun. 72*, 1153–1159.
53. Vinkler, C. (1981) *Biochem. Biophys. Res. Commun. 99*, 1095–1100.
54. Melandri, A. B., Fabbri, E., Firstater, E. & Melandri, B. A. (1975) *Biochim. Biophys. Acta, 376*, 72–81.
55. Farron, F. & Racker, E. (1970) *Biochemistry, 9*, 3829–3836.
56. McCarty, R. E. & Fagan, J. (1973) *Biochemistry, 42*, 1503–1507.
57. McCarty, R. E., Pittman, P. R. & Tsuchiga, Y. (1972) *J. Biol. Chem. 247*, 3048–3051.
58. Andreo, C. S. & Vallejos, R. H. (1976) *Biochim. Biophys. Acta, 423*, 590–601.

59. Moroney, J. V., Andreo, C. S., Vallejos, R. H. & McCarty, R. E. (1980) *J. Biol. Chem. 255*, 6670–6674.
60. Uribe, E. (1980) *Abstr. 5th Int. Congr. Photosynthesis*, p. 589.
61. Andreo, C. S., Ravizzini, R. A. & Vallejos, R. H. (1979) *Biochim. Biophys. Acta, 547*, 370–379.
62. Vallejos, R. H. & Andreo, C. S. (1976) *FEBS Lett. 61*, 95–99.
63. Vinkler, C. & Kornstein, R. (1982) *Proc. Natl Acad. Sci. USA, 79*, 3183–3187.
64. Klingenberg, M. (1977) *Eur. J. Biochem. 76*, 553–565.
65. Czeck, M. (1977) *Annu. Rev. Biochem. 46*, 359–384.

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